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14. ABSTRACT: The goal of this research is to define the molecular mechanisms responsible for the markedly increased risk of transformation to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) in patients with congenital neutropenia. We hypothesize that replicative stress and/or changes in the bone marrow microenvironment in patients with congenital neutropenia leads to a higher rate of accumulation of mutations in hematopoietic stem/progenitor cells (HSPCs), which, in turn, contributes to transformation to MDS/AML. We further hypothesize that G-CSF treatment accelerates the accumulation of mutations in HSPCs. Finally, we hypothesize that truncation mutations of <i>CSF3R</i> , which are common in patients with severe congenital neutropenia (SCN) and are associated with increased G-CSF signaling and transformation to MDS/AML, accentuate the rate of mutation accumulation. We will test these hypotheses in the following Specific Aims. <u>Aim 1. To determine whether HSPCs undergo premature genomic aging in SCN or SDS.</u> We will measure the mutation burden in individual HSPCs from patients with SCN, Shwachman-Diamond syndrome (SDS), cyclic neutropenia, or age-matched healthy controls. <u>Aim 2. To determine whether increased G-CSF signaling accelerates the mutation rate in HSPCs.</u> Here, we will assess the impact of prolonged (6 month) G-CSF therapy on HPSC mutation burden in mice. These data will provide novel insight into the mechanisms of leukemic transformation in CN. They also should provide new insight into the safety of long-term G-CSF therapy in CN. Finally, our novel assay to measure mutation burden in HSPCs may provide an approach to assess DNA damage after exposure to genotoxic agents, such as radiation.					
15. SUBJECT TERMS Congenital neutropenia; Severe congenital neutropenia; Shwachman Diamond syndrome; Cyclic neutropenia; Hematopoietic stem cells; Granulocyte colony-stimulating factor; Acute myeloid leukemia; Myelodysplastic syndrome					
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## 1. INTRODUCTION:

Severe congenital neutropenia (SCN) is a rare syndrome characterized by chronic neutropenia present from birth and recurring bacterial infections. Mutations of *ELANE* are the most common cause of SCN, accounting for approximately 50% of cases. Treatment with G-CSF is the standard of care for SCN, as it increases the level of circulating neutrophils and reduces infection-related mortality. Shwachman Diamond syndrome (SDS) is a recessive disorder characterized by exocrine pancreatic insufficiency, bone marrow dysfunction, and skeletal abnormalities. SDS is caused in most cases by bi-allelic mutations of *SBDS*. Current studies support a model of disease pathogenesis in which *SBDS* mutations lead to impaired ribosome assembly. A shared feature of SCN, SDS, and several other bone marrow failure syndromes that feature neutropenia is a marked propensity to develop a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). The cumulative incidence of MDS/AML in patients with SCN treated with G-CSF is 22%. Likewise, the cumulative incidence of MDS/AML in SDS is approximately 20%. Acquired gain-of-function mutations of *CSF3R*, encoding the G-CSF receptor, are present in approximately 40% of cases of SCN and are associated with the development of MDS/AML. Mutations of *RUNX1* are present in approximately 65% of patients with SCN who develop AML/MDS. In SDS, a recent study showed that mutations of *TP53* are common in MDS that develops in patients with SDS. Whether the *TP53* mutations represent an early, leukemia initiating event, or a late progression event (as has been shown for some types of secondary AML), is unknown. Despite these observations, the molecular mechanisms contributing to transformation to MDS/AML in congenital neutropenia syndromes are poorly understood, limiting the development of new therapies or strategies for risk stratification or early detection.

The accumulation of mutations in hematopoietic stem cells (HSCs) with age results in the production of a genetically heterogeneous cell population, with each HSC possessing its own unique set of private mutations. HSCs that acquire somatic mutations that confer a competitive fitness advantage relative to their normal counterparts may clonally expand. Indeed, several groups have documented the presence of clonal hematopoiesis in healthy individuals. Factors that increase the rate at which mutations accumulate in HSCs may increase the frequency of clonal hematopoiesis and ultimately MDS/AML. The common mutations causing SCN are not known to be directly involved in DNA repair, suggesting the possibility that non-cell autonomous mechanisms may contribute to the high rate of leukemic transformation. For example, granulocyte colony stimulating factor (G-CSF) expression is induced by neutropenia and may increase the rate at which HSCs accumulate mutations by inducing their replication. Of note, prior studies have demonstrated that the G-CSF receptor (*CSF3R*) is expressed on HSCs. Factors that select for HSCs carrying deleterious mutations also may increase the risk of MDS/AML. For example, we previously showed that HSCs carrying mutations in *TP53* are selected by exposure to chemotherapy. Thus, it is possible that HSC-cell autonomous and/or non-cell autonomous alterations in congenital neutropenia may confer a competitive fitness advantage to HSCs that carry leukemia-associated mutations. To test these possibilities, we measured the mutation burden in individual hematopoietic stem/progenitor cells (HSPCs) and characterized clonal hematopoiesis in patients with congenital neutropenia.

## 2. KEYWORDS:

Neutropenia  
Myelodysplastic syndrome  
Acute myeloid leukemia  
Severe congenital neutropenia  
Shwachman Diamond syndrome  
*TP53*  
Granulocyte colony-stimulating factor (G-CSF)  
Genomic sequencing  
Clonal hematopoiesis

### 3. ACCOMPLISHMENTS:

The major goals and objectives of this research remain the same as originally proposed. Progress and plans for each of the tasks proposed Statement of are detailed below.

**Task 1. To determine whether HSPCs undergo premature genomic aging in congenital neutropenia (Timeframe: 1-36 months).** In this task, we measured the mutation burden in individual hematopoietic stem/progenitor cell (HSPC) clones derived from healthy donors or patients with congenital neutropenia. We also used error-corrected sequencing approach on a panel of 46 genes to assess for clonal hematopoiesis. As detailed below, all experiments related to this task have been completed.

Our results show that mutation burden in HPSCs from patients with congenital neutropenia is similar to that seen age-matched healthy controls. Across all samples, the number of genic somatic mutations detected in the progeny of each HSPC ranged from 0-10. As reported previously, a strong correlation between HSPC mutation burden and the age of the patient was observed (Pearson  $r=0.83$ ,  $P<0.001$ ). The lowest number of mutations was present in the cord blood samples, with only  $1.4 \pm 0.29$  mutations per HSPC exome. The number of mutations detected in the exomes of HSPCs from healthy donors ( $3.9 \pm 0.38$ ) is similar to that observed from patients with SCN ( $3.6 \pm 1.2$ ) or SDS ( $1.8 \pm 0.65$ ). After adjusting for age, there was no difference in HSPC mutation burden in the different cohorts ( $P=0.34$  by analysis of covariance). Somatic copy number alterations were not identified in any of the hematopoietic colonies (data not shown). These data suggest that the rate at which mutations accumulate in HSPCs in patients with congenital neutropenia is not increased compared to that of healthy individuals.

We utilized a sensitive error-corrected sequencing approach to look for clonal hematopoiesis in the blood or bone marrow of patients with congenital neutropenia. Using this sequencing technique, we were able to reliably detect mutations with a variant allele frequency of at least 0.1%, corresponding to one cell in 500 carrying a mutation. We interrogated 46 genes that reported to be mutated in individuals with clonal hematopoiesis or MDS/AML. All of the patients with cyclic neutropenia or SCN (for whom information was available) were treated chronically with G-CSF, compared to 9 of 27 (33%) cases of SDS. Clonal hematopoiesis due to any mutation was identified in 5 of 17 (29%) healthy individuals, 5 of 13 (38%) patients with cyclic neutropenia, 25 of 40 (62%) SCN cases, and 16 of 27 (59%) of SDS cases ( $P=0.08$  by Pearson chi-square test of independence). Consistent with prior reports, clonal hematopoiesis due to mutations of *CSF3R* was detected in 40% (16 of 40) of patients with SCN, compared with 0 of 17 of healthy controls (Fisher's Exact  $P=0.003$ ). *CSF3R* mutations were detected in a single

patient with cyclic neutropenia (1/17, 7.7%,  $P=0.04$ ) and in no patients with SDS (0/27,  $P<0.001$ ). Of note, after removing *CSF3R* mutations, the percentage of cases with clonal hematopoiesis was similar between healthy controls and patients with SCN. Clonal hematopoiesis due to mutations of *TP53* was observed in 48% (13/27) of patients with SDS, but was not detected in 17 healthy donors ( $P<0.001$  by Fisher's Exact test). No mutations of *TP53* were detected in any of the patients with SCN (0/40,  $P<0.001$ ) or cyclic neutropenia (0/13,  $P=0.003$ ). After removing *TP53* mutations, the percentage of cases with clonal hematopoiesis was similar between healthy controls and patients with SDS. A manuscript detailing these findings has been accepted for publication in Blood.

1a. Obtain human studies approval for whole exome sequencing of healthy controls. Human studies approval at Washington University, University of Michigan, and the University of Washington have been obtained.

1b. Obtain human studies approval for studies from the DoD Human Research Projection Office. DOD approval has been obtained.

1c. Obtain human blood or bone marrow samples from patients with SDS, SCN, cyclic neutropenia, or healthy controls. We proposed to obtain a total of 15 bone marrow of blood samples each from patients SDS, SCN, cyclic neutropenia, or age-matched healthy controls. Except for cyclic neutropenia, we exceeded expectations for this task (see Table 1).

1d. Generate hematopoietic stem/progenitor cell (HSPC) colonies from patients with CN or healthy controls. We encountered difficulty in efficiently expanding hematopoietic colonies from the blood/bone marrow samples. This was particularly true for SDS, where only two SDS samples yielded hematopoietic colonies of sufficient size for exome sequencing. This consistent with prior studies showing impaired growth of SDS hematopoietic progenitors.

**Table 1. Human samples analyzed**

Sample type	Total # of samples	Successful HSPC expansion	Successful HSPC Sequencing	CHIP Sequencing
Normal	20	9	9	17
SCN	40	11	11	40
SDS	28	2	2	27
Cyclic	13	5	5	13

The total number of blood or bone marrow samples obtained is shown.

The number of successful HSPC expansions and HSPC clone sequencing is shown

The number of blood/bone marrow that underwent CHIP sequencing is shown

1e. Sequence HSPC clones and bone marrow fibroblasts. The number of samples were sequenced is shown in Table 1.

1f. Analysis of the sequence data. This task is completed.

1g. Validation of mutations. This task is completed.

**Task 2. To determine whether increased G-CSF signaling accelerates the mutation rate in HSPCs (Timeframe 1-21 months, completed).** In this task, we assessed the mutation burden in

individual murine HSPC clones exposed to G-CSF treatment for 6 months. These analyses were performed in wildtype and *Csf3r* mutant mice. Results are summarized in Figure 1.

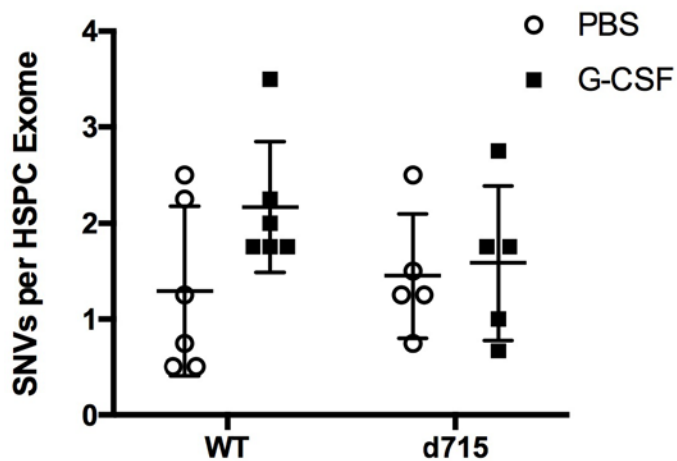
2a. Obtain regulatory approval from the DOD Animal Care and Use Review Office. DoD Animal Care approval has been obtained. This task is complete.

2c. Generate HSPC colonies from mice. We have successfully generated HSPC colonies from 27 wild-type or *Csf3r* mutant mice treated with G-CSF or saline alone for 6 months.

2d. Sequence HSPC clones. Sequencing of HSPC clones from a total of 22 mice is completed. Data are summarized in Figure 1.

2e. Analysis of the sequence data. This task is complete. Data are summarized in Figure 1.

2f. Validation of mutations. This task is complete. Data are summarized in Figure 1.



*Figure 1. Mutation burden in murine HSPCs.* Wild-type (WT) or *Csf3r*<sup>d715/d715</sup> (d715) mice were treated with pegylated G-CSF (1 mg/kg three times per week) for 6 months. Single Kit<sup>+</sup> lineage-hematopoietic stem/progenitor cells were sorted and expanded over a 3 week period with stromal cell support. The exomes of a minimum of 3 HSPC colonies along with matching tail DNA were sequenced. Shown is the average number of somatic single

nucleotide mutations or indels per exome per mouse (each data point represents the average of at least 3 HSPC colonies). Neither treatment with G-CSF nor the presence of truncating (activating) mutations of the G-CSF receptor (*Csf3r*) were associated with a difference in HSPC mutation burden.

#### 4. IMPACT

##### Impact on the development of the principal discipline:

Our data suggest that both HSPC-cell intrinsic and non-cell intrinsic changes may determine the competitive fitness of individual HSPCs. In the case of SCN, the persistently high levels of G-CSF drive the expansion of HSPCs carrying mutations of *CSF3R*. In SDS, impaired ribosome biogenesis induces p53-mediated growth inhibition, and drives expansion of HSPCs carrying *TP53* mutations. It is likely that additional stressors may influence the development of clonal hematopoiesis. Identifying cell-intrinsic and non-cell intrinsic stressors that shape the expansion of HSPCs may provide novel insights into the pathogenesis of AML or MDS.

Impact on other disciplines: The hematopoietic colony sequencing assay described here represents a method to assess mutation burden in hematopoietic stem/progenitor cells. This assay may have applications for individuals following exposure to genotoxic agents, such as radiation.

Impact on technology transfer: nothing to report

Impact on society beyond science and technology: nothing to report

## **5. CHANGES/PROBLEMS**

Changes in approach: nothing to report

Actual or anticipated problems: We encountered some difficulty in expanding hematopoietic colonies from patients with SDS. This is likely due to an inherent defect the proliferation of SDS HPSCs.

Significant changes in the use or care of human subjects, vertebrate animals, biohazards, and/or select agents: nothing to report

## **6. PRODUCTS**

### **Papers:**

Xia, J., Miller, C. A., Baty, J., Ramesh, A., Jotte, M. R. M., Fulton, R. S., Vogel, T. P., Cooper, M. A., Walkovich, K. J., Makaryan, V., *et al.* Somatic mutations and clonal hematopoiesis in congenital neutropenia. Blood, in press.

### **Abstracts:**

Xia, J., Shimamura A, Myers, K.C., Boxer L.A., Dale, D.C., Ramesh, A., Jotte, M., and Link, D. C. Mutation burden in hematopoietic stem cells is not increased in congenital neutropenia. Blood: 128:405, 2016

### **Presentations:**

Xia, J, American Society of Hematology, 58<sup>th</sup> Annual meeting, San Diego, CA, USA. December, 2016. "Mutation burden in hematopoietic stem cells is not increased in congenital neutropenia".

**Website(s) or other Internet site(s):** Nothing to report

**Technologies or techniques:** Nothing to report

**Inventions, patent applications, and/or licenses:** Nothing to report



## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals worked on this project:

Name:	Daniel C. Link
Project Role:	Principal Investigator
Researcher Identifier:	0000-0002-3410-3580

Nearest person month involved:	2 months
Contribution to Project:	no change
Funding Support:	no change

Name:	Jun Xia
Project Role:	Staff Scientist
Researcher Identifier:	None
Nearest person month involved:	6 months
Contribution to Project:	no change
Funding Support:	no change

Changes in active or other support of the PD/PI: Nothing to report

What other organizations are involved as partners:

Organization Name: The University of Washington  
Location of Organization: Seattle, Washington  
Partner's contribution to the project: Drs. Dale and Shimamura provide coded human blood or bone marrow samples from patients with congenital neutropenia.  
No changes

Organization Name: University of Michigan  
Location of Organization: Ann Arbor, MI  
Partner's contribution to the project: Dr. Larry Boxer provide coded human blood or bone marrow samples from patients with congenital neutropenia.  
No changes

**8. SPECIAL REPORTING REQUIREMENTS:** None

**9. APPENDICES:** None

## SOMATIC MUTATIONS AND CLONAL HEMATOPOIESIS IN CONGENITAL NEUTROPENIA

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## KEYPOINTS

- Hematopoietic stem/progenitor mutation burden is not increased in severe congenital neutropenia
- Clonal hematopoiesis due to mutations of *TP53* are present in the majority of patients with Shwachman Diamond syndrome

## ABSTRACT

Severe congenital neutropenia (SCN) and Shwachman-Diamond syndrome (SDS) are congenital neutropenia syndromes with a high rate of leukemic transformation. Hematopoietic stressors may contribute to leukemic transformation by increasing the mutation rate in hematopoietic stem/progenitor cells (HSPCs) and/or by promoting clonal hematopoiesis. We sequenced the exome of individual hematopoietic colonies derived from 13 patients with congenital neutropenia to measure total mutation burden and performed error-corrected sequencing on a panel of 46 genes on 80 patients with congenital neutropenia to assess for clonal hematopoiesis. An average of  $3.6 \pm 1.2$  somatic mutations per exome were identified in HSPCs from patients with SCN compared to  $3.9 \pm 0.4$  for healthy controls ( $p=NS$ ). Clonal hematopoiesis due to mutations in *TP53* were present in 48% (13/27) of patients with SDS but were not seen in healthy controls (0/17,  $p<0.001$ ) or patients with SCN (0/40,  $p<0.001$ ). Our SDS cohort was young (median age 6.3 years) and many of the patients had multiple *TP53* mutations. Conversely, clonal hematopoiesis due to mutations of *CSF3R* were present in patients with SCN but were not detected in healthy controls or patients with SDS. These data show that hematopoietic stress, including G-CSF, does not increase the mutation burden in HSPCs in congenital neutropenia. Rather, distinct hematopoietic stressors result in the selective expansion of HSPCs carrying specific gene mutations. In particular, in SDS there is enormous selective pressure to expand *TP53*-mutated HSPCs, suggesting that acquisition of *TP53* mutations is an early, likely initiating event, in the transformation to MDS/AML in patients with SDS.

## INTRODUCTION

Severe congenital neutropenia (SCN) is a rare syndrome characterized by chronic neutropenia present from birth and recurring bacterial infections. Mutations of *ELANE* are the most common cause of SCN, accounting for approximately 50% of cases, with mutations of *HAX1* and *G6PC3* accounting for an additional 10-20% of cases.<sup>1-4</sup> Treatment with G-CSF is the standard of care for SCN, as it increases the level of circulating neutrophils and reduces infection-related mortality.<sup>5</sup> Shwachman Diamond syndrome (SDS) is a recessive disorder characterized by exocrine pancreatic insufficiency, bone marrow dysfunction, and skeletal abnormalities. SDS is caused in most cases by bi-allelic mutations of *SBDS*.<sup>6</sup> Current studies support a model of disease pathogenesis in which *SBDS* mutations lead to impaired ribosome assembly.<sup>7-9</sup>

A shared feature of SCN, SDS, and several other bone marrow failure syndromes that feature neutropenia is a marked propensity to develop a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). The cumulative incidence of MDS/AML in patients with SCN treated with G-CSF is 22%.<sup>10</sup> Of note, MDS/AML has been reported in SCN secondary to *ELANE*, *HAX1*, *G6PC3*, or *WAS* mutations.<sup>11</sup> The cumulative incidence of MDS/AML in SDS is approximately 20%.<sup>12</sup> Acquired gain-of-function mutations of *CSF3R*, encoding the G-CSF receptor, are present in approximately 40% of cases of SCN and are associated with the development of MDS/AML.<sup>13,14</sup> Mutations of *RUNX1* are present in approximately 65% of patients with SCN who develop AML/MDS.<sup>15</sup> In SDS, a recent study showed that mutations of *TP53* are common in MDS that develops in patients with SDS.<sup>16</sup> Whether the *TP53* mutations represent an early, leukemia initiating event, or a late progression event (as has been shown for some types of secondary AML),<sup>17</sup> is unknown. Despite these observations, the molecular mechanisms contributing to transformation to MDS/AML in congenital neutropenia syndromes are poorly understood, limiting the development of new therapies or strategies for risk stratification or early detection.

The accumulation of mutations in hematopoietic stem cells (HSCs) with age results in the production of a genetically heterogeneous cell population, with each HSC possessing its own unique set of private mutations.<sup>18</sup> HSCs that acquire somatic mutations that confer a competitive fitness advantage relative to their normal counterparts may clonally expand. Indeed, several groups have documented the presence of clonal hematopoiesis in healthy individuals.<sup>19-21</sup> Factors that increase the rate at which mutations accumulate in HSCs may increase the frequency of clonal hematopoiesis and ultimately MDS/AML. The common mutations causing SCN are not known to be directly involved in DNA repair, suggesting the possibility that non-cell autonomous mechanisms may contribute to the high rate of leukemic transformation. For example, granulocyte colony stimulating factor (G-CSF) expression is induced by neutropenia and may increase the rate at which HSCs accumulate mutations by inducing their replication.<sup>22</sup> Of note, prior studies have demonstrated that the G-CSF receptor (CSF3R) is expressed on HSCs.<sup>23</sup> Factors that select for HSCs carrying deleterious mutations also may increase the risk of MDS/AML. For example, we previously showed that HSCs carrying mutations in *TP53* are selected by exposure to chemotherapy.<sup>24</sup> Thus, it is possible that HSC-cell autonomous and/or non-cell autonomous alterations in congenital neutropenia may confer a competitive fitness advantage to HSCs that carry leukemia-associated mutations. To test these possibilities, we measured the mutation burden in individual hematopoietic stem/progenitor cells (HSPCs) and characterized clonal hematopoiesis in patients with congenital neutropenia.

## **METHODS**

### **Human Subjects**

A total of 101 human blood or bone marrow samples were obtained for our study. These samples were divided into 5 cohorts: patients with SCN (40), patients with SDS (28), patients with cyclic neutropenia (13), healthy volunteers (17) and cord blood (3). Coded blood or bone

marrow samples from patients with congenital neutropenia were obtained from the Severe Chronic Neutropenia International Registry (<https://depts.washington.edu/registry/>), the SDS Registry (<http://sdsregistry.org>), or from various other academic institutions. Anonymized cord blood samples were obtained from the Saint Louis Cord Blood Bank (<http://www.slcbb.org>). Coded blood or bone marrow samples were obtained from healthy volunteers, with the following exclusion criteria: a personal history of cancer, the use of cytotoxic drugs for non-malignant disease, a history of radiation therapy, or known infections with Hepatitis B or C, HTLV, or HIV. In each case, the banking and distribution of these samples were approved by institutional review boards at the involved institution; written informed consent was obtained from all participants.

### **Hematopoietic progenitor cell expansion**

The low-density mononuclear cell fraction was isolated from peripheral blood or bone marrow by centrifugation at 400 x g for 30 minutes over a Histopaque 1077 gradient (Sigma), in some cases, red blood cells were lysed by incubating in Tris-buffered ammonium chloride (pH 7.4). An aliquot ( $1 \times 10^6$ ) of unselected mononuclear cells was removed and genomic DNA prepared using the QIAmp DNA Mini kit (Qiagen), per the manufacturer's instructions. The remainder of the cells were stained with a panel of fluorescein-conjugated lineage markers (CD3, CD19, CD14, and CD16), phycoerythrin-conjugated CD34, and allophycocyanin-conjugated CD38. CD34<sup>+</sup> CD38<sup>-</sup> lineage<sup>-</sup> cells were sorted at 1 cell per well into a 96-well plate using a Cytomation MoFlow or Sony Synergy cell sorter. Prior to sorting, the 96 well plate was seeded with irradiated (2000 cGy) AFT024 stromal cells (ATCC: SCRC-1007) at a density of  $2.5 \times 10^4$  cell per well). CD34<sup>+</sup> CD38<sup>-</sup> lineage<sup>-</sup> cells were cultured for 2-3 weeks in Iscove's Modified Dubelcco's media supplemented with 10% fetal calf serum, 1 mM L-glutamine, and the following human recombinant cytokines: stem cell factor (10 ng/ml), FLT3 ligand (25 ng/ml), thrombopoietin (20 ng/ml), interleukin-3 (10ng/ml), and granulocyte colony-stimulating factor (10

ng/ml). The cultures were maintained at 37°C with 5% CO<sub>2</sub> and ambient oxygen and supplemented with fresh media every 5 days. After 2-3 weeks of culture, wells with visible hematopoietic cell growth were expanded into a 24-well plate without AFT024 feeder cells for another week. Hematopoietic cells from the colonies were then harvested, counted, and genomic DNA prepared using the Qiagen QIAmp DNA Micro Kit (Qiagen).

### **HSPC clone whole exome sequencing and variant calling**

Genomic DNA was fragmented and exome capture was performed using a customized version of the Agilent SureSelect Human All Exon v2 kit, which targets 99.01% of CCDS exons, 93.29% of RefSeq genes, and 90% of known miRNA genes (MiRbase 14). Median sequence coverage was 104X (range: 46.2-232X). Sequence was aligned to reference sequence build GRCh37-lite-build37, as previously described.<sup>25</sup> Putative somatic mutations were identified via direct pairwise comparisons of each HSPC clone to the unfractionated total peripheral blood/bone marrow cells from which it was derived. Single nucleotide variants (SNVs) and insertions/deletions (Indels) were detected as previously described.<sup>25</sup> SNVs and Indels that exceeded 0.1% frequency in the 1000 genomes or NHLBI exome sequencing projects were removed. To remove non-clonal events, reference and variant read counts were compared to a binomial distribution of the same number of reads, assuming a variant allele frequency of 50%. Sites that significantly differed (Fisher's exact test  $P > 0.05$ ) from the expected binomial distribution were removed. Additional filters required the number of reference reads in the control sample to be greater than 30, the number of variant reads in the HSPC clone sample to be greater than 5, and the variant allele frequency in the control sample to be less than 3%. Finally, sites were manually reviewed to remove other classes of alignment artifacts.

### **Error-corrected (Haloplex) targeted sequencing and variant detection**

Error-corrected sequencing was performed using the Agilent HaloPlex HS Target Enrichment System, as previously described.<sup>26</sup> A customized HaloPlex HS Target Enrichment assay targeting 46 genes mutated in clonal hematopoiesis and/or MDS/AML (**Supplemental Table 1**) was designed using the Agilent SureDesign platform. The probes had dual indices: a unique molecular barcode to allow for error-corrected sequencing and a sample index to allow for sample multiplexing. 500 ng of genomic DNA was hybridized to the custom probes, ligated, captured with streptavidin, and PCR amplified (x 24 cycles) to create read families each with its own molecular bar code. Median sequence coverage was 17,824X (range 9,491-34,895). Variants were required to be supported by three read families. Filters were applied to remove artifacts appearing at homopolymer runs of length greater than 4 and alignment artifacts appearing in greater than 5% of a panel of normal samples. Next, background noise calculation was performed on a position-by-position basis for each identified variant as follows: For each variant, readcounts were gathered from all other samples, excluding those sites with variant allele frequency above 25%, which were assumed to be germline SNPs. In a single case (SCN51), we retained a truncation *CSF3R* mutations with a variant allele frequency of 39%. A p-value was obtained via Fisher's exact test, comparing the reference and variant reads at a site to the number of reference and variant reads at that site in all other samples. Multiple testing correction was applied with the p value adjust function (default parameters). Those variants with an adjusted p-value of less than 0.1 were retained. The same process is repeated with subsequent background calculations excluding all variants retained in previous rounds until no new variants were identified. Finally, we interrogated the Exome Aggregation Consortium database (<http://exac.broadinstitute.org>) to remove variants with an adjusted allele frequency <0.0005. Following manual review, we reported somatic variants that generated missense, nonsense, or splice site mutations.

## Statistical Analysis



Continuous and count variables are described with means and standard deviations when distributions appear symmetrical and by medians when the distributions appear skewed. Categorical variables are described by percentages. Pearson correlations are used when the distributions of variables appear symmetrical and Spearman correlations when they appear asymmetrical or there are outlying values that would inflate a Pearson correlation. Independence between dichotomous variables in two-way tables is evaluated with Fisher's Exact tests while exact chi-square tests are used in two-way tables with small sample sizes where one or both variables are not dichotomous. Independent-sample t-tests are used to compare two groups with respect to symmetrically distributed continuous variables. Analysis of covariance is used as an alternative to ANOVA in order to adjust for confounding variables such as age. The Kruskal-Wallis nonparametric alternative to ANOVA is used when there are multiple groups and the dependent variable is asymmetric. The analysis is followed by Bonferroni-adjusted pairwise Wilcoxon tests. Logistic regression is used when the dependent variable is dichotomous with results reported as odds ratios with 95% confidence intervals. For all tests, a P-value of less than 0.05 was used to indicate statistical significance. Statistical analyses were done with SAS Version 9.4 for Windows and the R statistical package.

## **RESULTS**

### **Hematopoietic progenitor mutation burden**

To determine whether chronic neutropenia and/or long-term treatment with G-CSF increased mutation burden in hematopoietic progenitors, we sequenced the exomes of individual, ex-vivo expanded HSPCs from patients with congenital neutropenia (Fig. 1A). These sequences were compared with the exome sequence of total leukocytes from the same patient to identify clonal somatic mutations (see Methods). Whereas we were successful in generating hematopoietic colonies of sufficient size for exome sequencing from the majority of SCN, healthy donors, and cord blood samples, only 2 of 15 (13.3%) SDS samples generated such colonies. This is

consistent with a study showing that HSPCs from patients with SDS have impaired growth.<sup>27</sup> In total, we sequenced hematopoietic colonies from 11 patients with SCN, 2 with SDS, 6 healthy donors, and 3 umbilical cord blood samples; patients with evidence of MDS or AML at the time of tissue collection were excluded (Table 1 and Supplemental Tables 2 & 3). All of the patients with SCN for whom information was available carried heterozygous germline mutations of *ELANE* and were being treated chronically with G-CSF. Of note, the two patients with SDS were younger than those in the SCN cohort, and had not received prior G-CSF treatment.

Across all samples, the number of genic somatic mutations detected in the progeny of each HSPC ranged from 0-10 (Fig. 1B and Supplemental Table 4). As reported previously, a strong correlation between HSPC mutation burden and the age of the patient was observed (Pearson  $r=0.83$ ,  $P<0.001$ ) (Fig. 1C).<sup>18</sup> The lowest number of mutations was present in the cord blood samples, with only  $1.4 \pm 0.29$  mutations per HSPC exome (Fig. 1D). The number of mutations detected in the exomes of HSPCs from healthy donors ( $3.9 \pm 0.38$ ) is similar to that observed from patients with SCN ( $3.6 \pm 1.2$ ) or SDS ( $1.8 \pm 0.65$ ). After adjusting for age, there was no difference in HSPC mutation burden in the different cohorts ( $P=0.34$  by analysis of covariance). Somatic copy number alterations were not identified in any of the hematopoietic colonies (data not shown). These data suggest that the rate at which mutations accumulate in HSPCs in patients with congenital neutropenia is not increased compared to that of healthy individuals.

## Clonal Hematopoiesis

We utilized a sensitive error-corrected sequencing approach to look for clonal hematopoiesis in the blood or bone marrow of patients with congenital neutropenia. Using this sequencing technique, we were able to reliably detect mutations with a variant allele frequency of at least 0.1%, corresponding to one cell in 500 carrying a mutation. We interrogated 46 genes that have been reported to be mutated in individuals with clonal hematopoiesis or

MDS/AML (Supplemental Table 1). We analyzed 17 healthy individuals, 40 with SCN, and 27 with SDS (Table 2 and Supplemental Tables 2 & 3). We also analyzed 13 patients with cyclic neutropenia. Cyclic neutropenia is characterized by intermittent neutropenia and is caused, in most cases, by mutations of *ELANE*. However, in contrast to SCN, it rarely transforms to MDS/AML.<sup>28</sup> All of the patients with cyclic neutropenia or SCN (for whom information was available) carried heterozygous germline mutations of *ELANE*, and all of the patients with SDS carried biallelic germline mutations of *SBDS*. As expected, the baseline (pre-G-CSF treatment) absolute neutrophil count (ANC) was significantly lower in patients with SCN compared to patients with SDS or cyclic neutropenia ( $P < 0.001$  and  $P < .01$ , respectively, using Bonferroni-corrected Wilcoxon tests). All of the patients with cyclic neutropenia or SCN (for whom information was available) were treated chronically with G-CSF, compared to 9 of 27 (33%) cases of SDS. Ten of 40 patients (25%) of patients with SCN underwent allogeneic stem cell transplantation, compared to a single patient with cyclic neutropenia, and one patient with SDS. Only two patients with SCN in our cohort are known to have developed AML or MDS.

Clonal hematopoiesis due to any mutation was identified in 5 of 17 (29%) healthy individuals, 5 of 13 (38%) patients with cyclic neutropenia, 25 of 40 (62%) SCN cases, and 16 of 27 (59%) of SDS cases ( $P = 0.08$  by Pearson chi-square test of independence) (Fig. 2A, and Supplemental Table 5). No consistent effect of tissue origin (i.e., blood versus bone marrow) was observed on the incidence of clonal hematopoiesis (Supplementary Figure S1A). Although clonal hematopoiesis was not detected in the three cord blood samples tested, we observed no difference in the median age of individuals with or without clonal hematopoiesis, which may reflect the rather narrow age range of cases analyzed in this study (Fig. 2B).

### **Clonal Hematopoiesis with *CSF3R* mutations**

Consistent with prior reports,<sup>14,29</sup> clonal hematopoiesis due to mutations of *CSF3R* was detected in 40% (16 of 40) of patients with SCN, compared with 0 of 17 of healthy controls

(Fisher's exact  $P=0.003$ , Fig. 2C). *CSF3R* mutations were detected in a single patient with cyclic neutropenia (1/17, 7.7%,  $P=0.04$ ) and in no patients with SDS (0/27,  $P<0.001$ ). Of note, after removing *CSF3R* mutations, the percentage of cases with clonal hematopoiesis was similar between healthy controls and patients with SCN (Fig. 2D). The size of the hematopoietic clone carrying a *CSF3R* mutation ranged from 0.26% to 78% of cells in the blood samples (median 0.70%). The number of *CSF3R* mutations per patient with SCN ranged from 0-3 (Fig. 2E). Consistent with prior reports, all of the *CSF3R* variants are nonsense mutations that truncate the cytoplasmic domain of the G-CSF receptor (Fig. 2F). Logistic regression, both adjusted for age and unadjusted, showed that the presence of *CSF3R* mutations was not related to standard measures of disease severity in SCN, including baseline absolute neutrophil count or median dose of G-CSF (Figs. 2G & H).

### **Clonal Hematopoiesis with *TP53* mutations**

Clonal hematopoiesis due to mutations of *TP53* was observed in 48% (13/27) of patients with SDS, but was not detected in 17 healthy donors ( $P<0.001$  by Fisher's exact test, Fig. 3A). No mutations of *TP53* were detected in any of the patients with SCN (0/40,  $P<0.001$ ) or cyclic neutropenia (0/13,  $P=0.003$ ). *TP53* mutations were detected at similar frequency in blood and bone marrow samples from patients with SDS (Supplementary Figure S1B). After removing *TP53* mutations, the percentage of cases with clonal hematopoiesis was similar between healthy controls and patients with SDS (Fig. 3B). A significant relationship between the presence of *TP53* mutations and age was observed with an odds ratio of 1.53 (95% confidence interval: 1.12-2.09,  $P<0.01$ ) (Fig. 3C). Remarkably, 80% (8/10) of patients  $\geq 10$  years of age had at least one *TP53* mutation, including one patient with 8 different *TP53* mutations. The *TP53* mutations were present in a low percentage of bone marrow cells (median 0.76%, range 0.1-7.7%). For one case (SDS34), multiple bone marrow samples were analyzed and showed that an *R249Q TP53* mutation not detected at age 4, was present at a variant allele fraction of 1.1%

at age 6.2 years, with an increase to 3.2% one month later (Fig. 3D). The *TP53* mutations clustered in the DNA binding domain of the gene, and all were present in the International Agency of Research on Cancer TP53 Database (Fig. 3E). Logistic regression after adjusting for age showed that the presence of *TP53* mutations was not related to the degree of neutropenia, G-CSF treatment, or presence of anemia or thrombocytopenia (Figs. 3F-H and data not shown).

## DISCUSSION

In this report, we show that the mutation burden in HSPCs from patients with SCN is comparable to that of age-matched health individuals. Due to the limited number of SDS samples analyzed, firm conclusions about mutation burden in SDS HSPCs is not possible. Nonetheless, these data suggest that an elevated mutation rate in HSPCs is not solely responsible for the marked increased risk of MDS/AML in congenital neutropenia. Although the published data are limited, this conclusion is supported by reports showing that the overall mutation burden in MDS or AML arising in the setting of SCN or SDS is comparable to that of de novo MDS/AML.<sup>16,30</sup>

Here, we provide evidence for the selective expansion of HSPCs carrying specific gene mutations in congenital neutropenia. In SDS, nearly 50% of patients have clonal hematopoiesis due to mutations of *TP53*. Multiple *TP53* mutations per patient were often detected. Of note, the variant allele frequencies of the *TP53* mutations in a given patient often varied considerably. For example, in patient SDS31, in whom 8 different *TP53* mutations were identified, the variant allele frequency ranged from 0.12 to 3.0%. Although single cell genotyping is needed to confirm, this observation suggests that in patients with multiple *TP53* mutations, the mutations likely arose in distinct HSPCs. The frequency of *TP53* mutations increase with age in patients with SDS and were not seen in any patients with SCN or cyclic neutropenia, or healthy controls. These observations show that specific stressors are present in SDS that strongly and specifically select for HSPCs carrying *TP53* mutations.

Mutations of *SBDS* that are present in the great majority of cases of SDS result in impaired ribosome biogenesis.<sup>8,9,31</sup> There is evidence that ribosome biogenesis stress induces p53 expression, which in turn, results in growth arrest. For example, mutations in genes encoding for ribosomal proteins RPS19 in Diamond-Blackfan Syndrome or RPS14 in 5q-syndrome result in impaired ribosome biogenesis and induction of *TP53* expression in erythroid progenitors.<sup>32,33</sup> Importantly, genetic or pharmacologic inhibition of p53 rescues the defect in erythropoiesis in RPS19-deficient cells, establishing the importance of increased *TP53* expression in these disorders.<sup>34-36</sup> Increased p53 expression also has been identified in hematopoietic cells from patients with SDS or in *Sbds* deficient murine hematopoietic cells.<sup>31,37</sup> Moreover, in mice with targeted disruption of *Sbds* in pancreatic cells, genetic ablation of *Trp53* rescues the severe atrophy in pancreatic acinar cells.<sup>38</sup> Together, these observations suggest a model in which elevated p53 expression due to ribosome biogenesis stress in SDS HSPCs results in impaired HSPC growth and/or survival. Mutations of *TP53* in HSPCs are predicted to attenuate this growth arrest, resulting in their selective expansion in patients with SDS. Our data suggest that the acquisition of *TP53* mutations is an early, initiating event, for the transformation to MDS/AML in SDS patients. Consistent with this conclusion, a recent study showed that 7 of 7 (100%) of cases of MDS arising in the setting of SDS carried *TP53* mutations.<sup>16</sup> However, since nearly all of the older patients with SDS had at least one *TP53* mutation, other mutations, including mutations of the residual *TP53* allele, are likely to be required for leukemic transformation. The predictive value of clonal hematopoiesis due to *TP53* mutations for the development of MDS/AML in patients with SDS is currently unclear. However, 8 of 10 (80%) of our SDS patients >10 years had at least one *TP53* mutation. Thus, it is unlikely that the simple presence of *TP53* mutations in blood/bone marrow will be a useful biomarker for the development of MDS/AML in these patients. Whether the number of *TP53* mutations, maximum variant allele fraction, and/or increase in *TP53* allele burden over time are predictive will require prospective longitudinal studies.

We confirm prior studies showing a high incidence of *CSF3R* truncation mutations in patients with SCN.<sup>13,14,29,39</sup> The truncated G-CSF receptor, while remaining dependent on G-CSF, transmits a sustained, increased signal in response to G-CSF<sup>40-42</sup>. Expression of the truncated G-CSF receptor confers a competitive advantage to HSCs in mice that is dependent on chronic G-CSF treatment<sup>43</sup>. Together, these observations suggest that the very high level of G-CSF present in patients (either through endogenous production or pharmacologic administration) is driving the expansion of HSPCs carrying *CSF3R* mutations. Of note, no increase in clonal hematopoiesis due to other gene mutations was observed, demonstrating the highly selective nature of *CSF3R*-dependent clonal expansion in SCN. There is evidence that *CSF3R* mutations contribute to the development of MDS/AML. A study showed that 13 of 18 (72%) patients with SCN who developed MDS/AML carried *CSF3R* mutations, compared with 43 of 125 (34%) without MDS/AML.<sup>14</sup> Moreover, truncation mutations of *Csf3r* cooperate with the *PML-RAR* oncogene to induce AML in mice.<sup>44</sup> On the other hand, there are reports of MDS/AML arising in patients with SCN prior to the availability of G-CSF.<sup>45-47</sup> The predictive value of *CSF3R* mutations in patients with SCN for MDS/AML is uncertain. Our study does not resolve this issue, since only two patients in our SCN cohort are known to have developed MDS/AML. Of note, prior studies show that *CSF3R* mutations can persist for many years (and occasionally disappear) without developing MDS/AML in some patients with SCN.<sup>14,30</sup> A recent report showed that mutations of *RUNX1* were present in the majority (64.5%) of patients with SCN who developed MDS or AML.<sup>15</sup> Interestingly, we identified no *RUNX1* mutations in our SCN cohort, which includes two patients who later developed AML. This is consistent with the conclusion by Skokowa et al that *RUNX1* mutations are a late step in leukemic transformation in patients with SCN.<sup>15</sup>

Our study has several limitations. Most importantly, the number of samples in which HSPC mutation burden was determined, especially for SDS, was small. Only a minority of SDS samples generated hematopoietic colonies of sufficient size for analysis, and it is not clear

whether these cases are representative of SDS in general. Our studies of clonal hematopoiesis were limited to a panel of 46 genes; it is possible that mutations in other genes contribute to clonal hematopoiesis in congenital neutropenia.

In summary, our data suggest that both HSPC-cell intrinsic and non-cell intrinsic changes may determine the competitive fitness of individual HSPCs. In the case of SCN, the persistently high levels of G-CSF drive the expansion of HSPCs carrying mutations of *CSF3R*. In SDS, impaired ribosome biogenesis induces p53-mediated growth inhibition, and drives expansion of HSPCs carrying *TP53* mutations. It is likely that additional stressors may influence the development of clonal hematopoiesis. For example, a recent study reported that exposure to chemotherapy results in a higher incidence of clonal hematopoiesis carrying *TP53* or *PPM1D* mutations.<sup>48</sup> Identifying cell-intrinsic and non-cell intrinsic stressors that shape the expansion of HSPCs may provide novel insights into the pathogenesis of AML or MDS.

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## **AUTHORSHIP CONTRIBUTIONS**

JX and DCL wrote the paper, designed and performed the research, and analyzed and interpreted the data; JX, AR and MRMJ processed samples; CAM and RSF provided bioinformatics support for the sequence data analysis; JB provided statistical support for data analyses; TPV, MAC, KJW, LAB, VM, AAB, DCD, MCD, DBW, AV, KCM, RJR, AAB and AS provided samples for this study.



## CONFLICT OF INTEREST DISCLOSURES

The authors have no conflicts of interest to report.

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## Figure Legends

**Figure 1. Hematopoietic progenitor mutation burden.** (A) experimental schema. CD34<sup>+</sup> CD38<sup>-</sup> lineage- cells from blood (Nm1, Nm4, and cord blood) or bone marrow were sorted one cell per well and expanded on stromal support for 2-4 weeks. Exome sequencing was performed on 4 hematopoietic progenitor clones isolated from 6 healthy donors, 3 cord blood, 11 severe congenital neutropenia (SCN), or 2 Shwachman-Diamond syndrome (SDS) patients. Somatic mutations were identified by comparison to exome sequence data from matched unfractionated blood or bone marrow leukocytes. (B) the number of somatic SNVs per exome for each clone. ND: not determined. (C) the average number of somatic SNVs per hematopoietic progenitor exome versus age at sample collection. (D) the average number of somatic SNVs per exome. The mean  $\pm$  SEM is shown.

**Figure 2. Clonal hematopoiesis with CSF3R mutations.** (A) Percentage of cases with clonal hematopoiesis due to any gene mutation. (B) Age of individuals with or without clonal hematopoiesis due to any gene mutation. (C) Percentage of cases with clonal hematopoiesis due to *CSF3R* mutations. \*\*\*P < 0.001 compared to healthy donors. (D) Percentage of cases with clonal hematopoiesis due to mutations in genes besides *CSF3R*. (E) Age of patients with SCN based on the number of *CSF3R* mutations. (F) The *CSF3R* mutations, with the number of times the mutation was seen in parentheses. IG: immunoglobulin-like domain; CRH: cytokine receptor-homologous domain; FNIII: fibronectin type III domains; TM: transmembrane domain; CT: cytoplasmic domain. (G) Absolute neutrophil count (ANC) prior to G-CSF treatment. (H) The median G-CSF dose. The mean  $\pm$  SD is shown.

**Figure 3. Clonal hematopoiesis with TP53 mutations.** (A) Percentage of cases with clonal hematopoiesis due to *TP53* mutations. (B) Percentage of cases with clonal hematopoiesis due to mutations in genes besides *TP53*. \*\*\*P < 0.001 compared to healthy donors. (C) Number of

*TP53* mutations per patient with SDS versus age. (D) Variant allele frequency (VAF) for *R249Q* *TP53* in serial bone marrow samples obtained for patient SDS34. (E) The *TP53* mutations, with the number of times the mutation was seen in parentheses. (F) Number of patients with severe neutropenia (ANC < 500 per mm<sup>3</sup>), mild/moderate neutropenia (ANC 500-1500 per mm<sup>3</sup>), or no neutropenia. (G) Number of patients with anemia. (H) Number of patients with thrombocytopenia.

**Table 1. Demographic and Disease Characteristics HPC Mutation Burden Cohort**

Characteristic	Normal (N = 6)	SCN (N = 11)	SDS (N = 2)
Age -- yr			
Mean (± SD)	17.7 ± 4.4	16.6 ± 7.9	7.0 ± 7.1
Median (range)	20 (9-20)	15.5 (6-36)	7.0 (2-12)
Female sex-- no (%)	2 (33.3)	5 (45.4)	1 (50)
G-CSF treatment--no (%) *	0 (0)	10 (100)	0 (0)
ANC per mm <sup>3</sup> †			
Mean (± SD)	na	62 ± 99	1170 ± 1198
Median (range)	na	0 (0-300)	1170 (330-2020)
<i>ELANE</i> mutation -- no (%)			
††	na	10 (100)	na
<i>SBDS</i> mutation -- no (%)	na	na	2 (100)

HPC: hematopoietic progenitor cell; na: not available

Absolute neutrophil count (ANC) obtained prior to G-CSF therapy is shown.

Percentage of patients with germline heterozygous *ELANE* or biallelic *SBDS* mutations is shown

\* Data not available for 1 patient with SCN

† Data not available for 2 patients with SCN

†† Data not available for 1 patient with SCN

**Table 2. Demographic and Disease Characteristics Clonal Hematopoiesis Cohort**

Characteristic	Normal (N = 17)	Cyclic (N = 13)	SCN (N = 40)	SDS (N = 27)
Age -- yr				
Mean ( $\pm$ SD)	17.2 $\pm$ 10.1	24.5 $\pm$ 14.1	11.6 $\pm$ 10.3	7.9 $\pm$ 5.0
Median (range)	17 (4-34)	26 (3-47)	10 (0.25-45)	6.3 (2-19)
Female sex-- no (%)	8 (47.0)	4 (30.7)	23 (57.5)	11 (37.9)
G-CSF treatment--no (%) *	0 (0)	10 (100)	38 (97.4)	10 (37.0)
ANC per mm <sup>3</sup> †				
Mean ( $\pm$ SD)	na	613 $\pm$ 554	140 $\pm$ 150	1080 $\pm$ 1070
Median (range)	na	400	90 (0-650)	770 (0-4490)
<i>ELANE</i> mutation -- no (%) ††	na	9 (100)	39 (100)	na
<i>SBDS</i> mutation -- no (%)	na	na	na	27 (100)
Allogenic stem cell transplant -- no (%)	na	1 (7.6%)	10 (25%)	1 (3.7)
AML or MDS -- no (%)^	na	0 (0)	2 (5.0%)	0 (0)

\* Data not available for 3 patients with cyclic neutropenia, 2 patients with SCN, and 2 patients with SDS.

† Absolute neutrophil count (ANC) obtained prior to G-CSF therapy is shown.

Data not available for 8 patients with cyclic neutropenia and 3 patients with SCN

†† Percentage of patients with germline heterozygous *ELANE* or biallelic *SBDS* mutations is shown

Data not available for 4 patients with cyclic neutropenia and 1 patient with SCN

^ Subsequent development of AML or MDS. No patient had MDS or AML at the time of sample analysis

na: not available



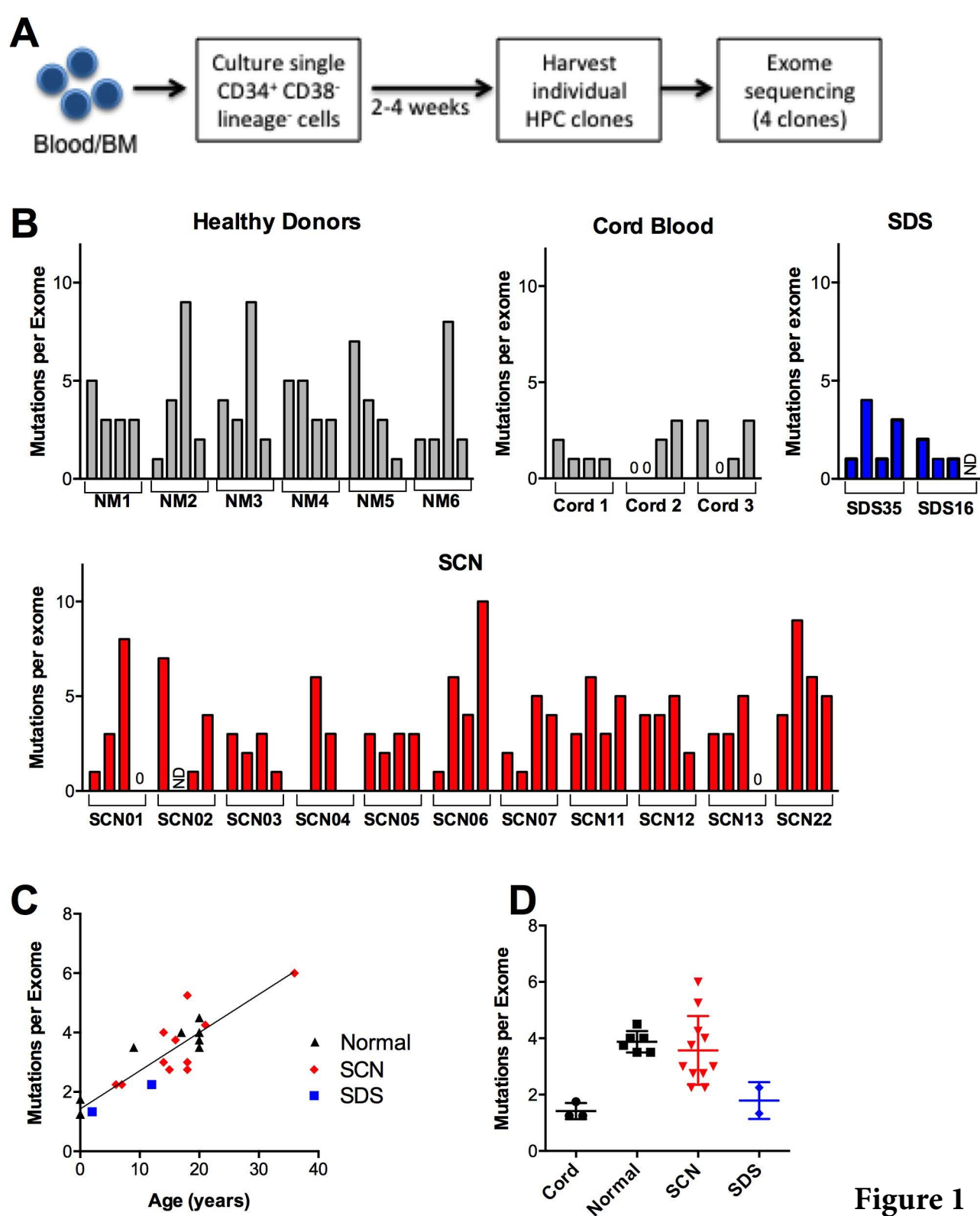
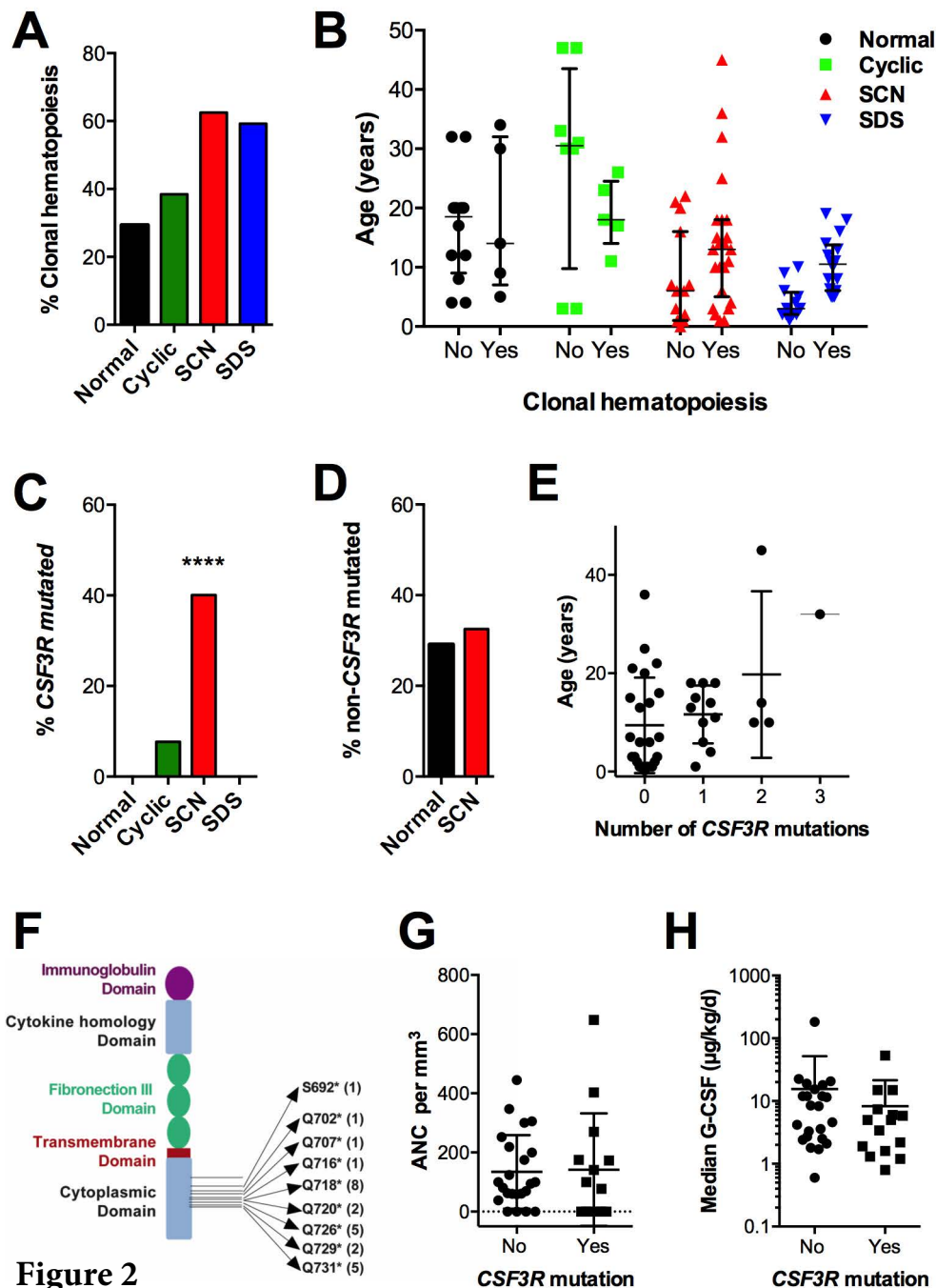


Figure 1



**Figure 2**

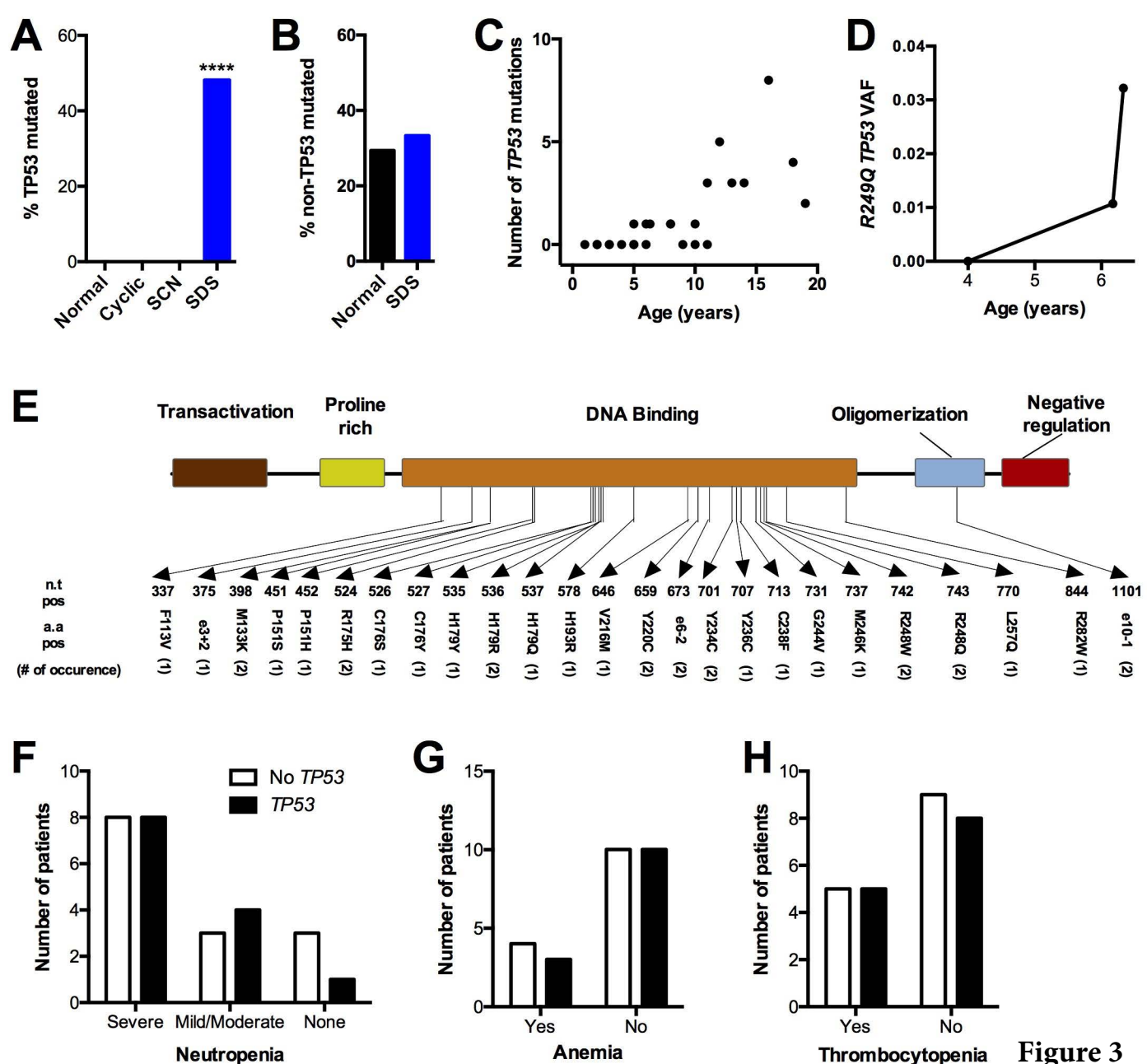


Figure 3